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The aim of this research is to determine the basis for molecular stability of the antioxidant enzymes, superoxide dismutase and catalase, from selected microorganisms and the contribution of these enzymes to the resistance to extreme and fluctuating UV radiation

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Grant Award NO.FA9550-09-1-0349
Report: Annual Technical Report
Period: 08/2011– 08/2012
August 30, 2012

Title: Extremophilic Enzymatic Response for Protection against UV-Radiation Damage.

Research Interest Category: Chemistry and Life Sciences, Biophysical Mechanisms.

Corresponding Program Manager: Dr. Hugh C. DeLong, hugh.delong@afosr.af.mil

Main Objective

The aim of this research is to determine the basis for molecular stability of the antioxidant enzymes, superoxide dismutase and catalase, from selected microorganisms and the contribution of these enzymes to the resistance to extreme and fluctuating UV radiation.

Note: This project started in August 2009.

Extremophiles in their natural environments are exposed to several conditions that favour ROS generation. Little is known about how these microorganisms can live under these conditions, in particular, what is the importance of superoxide dismutase and catalase in thermophiles, psychrophiles and halophiles exposed to high UV radiation.

To study the effects of key enzymes such **superoxide dismutase** and **catalase** as part of the antioxidant enzymatic system and the resistance of these enzymes to UV radiation, we selected two extremophilic microorganisms, recently isolated in our laboratory. One is a low temperature (2ºC), halophile isolated from Antarctica (I1p) and the other one a desiccation resistant thermophile (73ºC) from an sterilization oven, previously isolated and studied in the AFOSR project FA9550-06-1-0502 (microorganism E1).

The following objectives were proposed to develop during the project:

1.- To study the contribution of the antioxidant enzymes to the resistance against extreme and fluctuating UV radiation.
2.-To determine the basis for molecular stability of the antioxidant enzymes, superoxide dismutase and catalase, from two selected extreme microorganisms.
3.-To clone and express into *Escherichia coli* the genes that codify for the enzymes superoxide dismutase and catalase from the selected microorganisms to study protection effects of these genes when the *E. coli* strain is exposed to UV radiation.

This report is presenting the experimental results obtained during the development of the third and final objective of this project[1].

Specific Objective addressed in this Report:

Objective 3: To clone and express into Escherichia coli the genes that codify for the enzymes superoxide dismutase and catalase from the selected microorganisms to study protection effects of these genes when the E. coli strain is exposed to UV radiation.

[1] The successful results of the objectives one and two have been informed in the previous report.

1. Cloning and expression of Mn Superoxide dismutase gene from E1.

The experimental strategy designed for achieving this objective is explained in Figure 1. The aim was to clone and express the genes that codify for superoxide dismutase (SOD) in the microorganism E1.

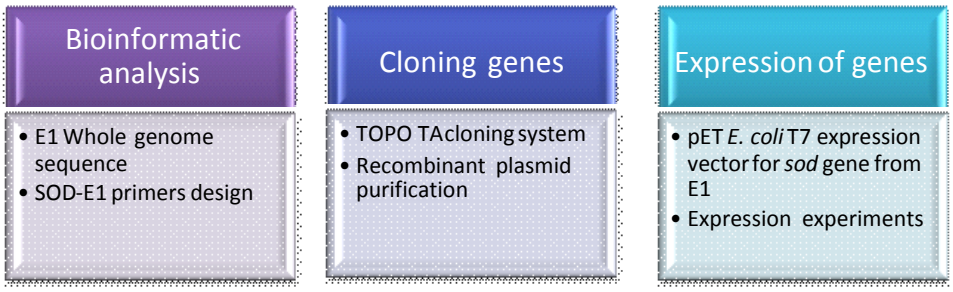


Figure 1. Experimental strategy for cloning and expression.

1.1 Whole genome sequence of E1.

After the genome of E1 was sequenced, we performed a bioinformatic analysis of the contigs. We found three different genes that codify for a Superoxide dismutase enzyme: Copper-Zinc-SOD, Manganese-SOD and Manganese-Iron-SOD. The following analysis and experiments are related to the Manganese-SOD enzyme, that we previously characterized during this project. We also performed a three dimensional structure prediction of E1 Manganese-SOD (Mn-SOD).

1.2 Three dimensional structure prediction of E1 superoxide dismutase

The protein sequence of Mn-SOD was used to perform a BLASTp against the PDB database in order to find templates suitable for comparative modeling. The best HIT in terms of sequence identity, similarity and coverage were considered for comparisons and validation of the model.

The Phyre2 web-server and database (Kelley *et al.,* 2009) were used to generate a model of the 3D structure of Mn-SOD. The obtained model was further validated using Prosa-web (Sippl *et al.,* 1993; Wiederstein *et al.,* 2007), proQ (Elofsson *et al.,* 2001) and Verify 3d (JU Bowie *et al.,* 1991). In all cases the model was compared to the structure of *Bacillus subtilis* SOD (pdb-id 2rcv), the best hit obtained from BLASTp.

The best hit in terms of sequence identity, similarity and coverage corresponds to the crystal structure of *Bacillus subtilis* SOD (pdb-id 2rcv) with an 80% of sequence identity, 90% of sequence similarity and 99% coverage. This structure was considered and used in further analysis as the reference template.

The Phyre2 web-server as mentioned above was used for the model generation. A set of 10 templates were considered for the modeling process, which posses a sequence identity with Mn-SOD of values between 50% and 80%. The procedure generated a final model in which 98% of the residues were modeled with more than 90% of accuracy.

The model generated with Phyre2 was evaluated with 3 standard tools: Prosa-web server, ProQ server and Verify3D. For comparison, and as stated in methods, the evaluation process considered a comparison with 2rcv. A very similar prosa z-score of -9.46 and -9.56 was obtained from the model and 2rcv respectively. The ProQ neural network provided an LG score of 5.637 and 5.215 for the Mn-SOD model and the 2rcv structure respectively. This method considers that models with LGscore>4 are extremely good. Regarding Verify 3D, both the model and 2rcv possesses more than 90% of the residues and had an averaged 3D-1D score > 0.2. All these results suggest that the fold of the Mn-SOD model is reliable and the model is suitable for further analysis and studies.

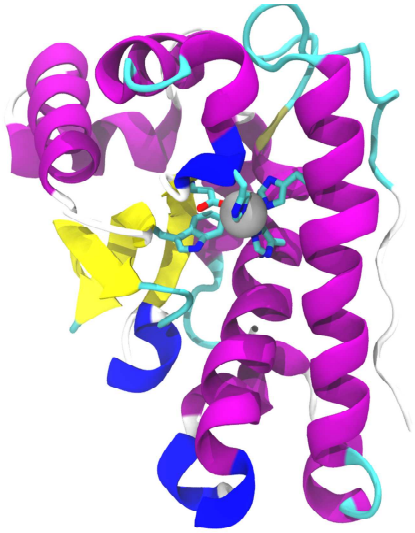


Figure 2. Ribbon representation of the E1-SOD model. The protein is colored by secondary structure elements (a-helix=purple, b-strand=yellow, 3-10 helix=blue, turn=cyan, coil=white). Relevant residues of the Mn binding site are shown in licorice representation and colored by element (carbon=cyan, nitrogen=blue, oxygen=red). A representative Mn atom is displayed in vdW representation. Figure 3 made with VMD (Humphrey et al., 1996).

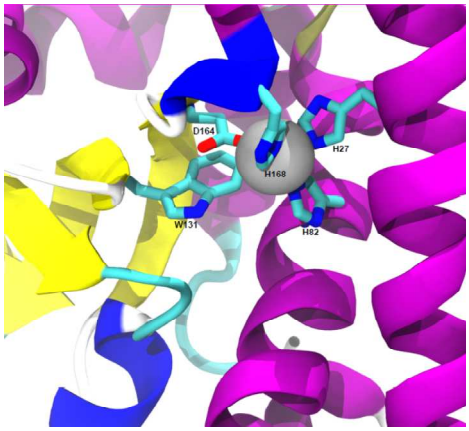


Figure 3. Close-up of the Mn binding site of the E1-SOD model. Relevant residues are shown in licorice representation and colored by element (carbon=cyan, nitrogen=blue, oxygen=red). A representative Mn atom displayed in vdW representation has been placed considering as reference the coordinates of the Mn atom found in 2rcv. Figure made with VMD (Humphrey et al., 1996).

1.3 Molecular analysis

The molecular strategy for cloning and expressing UV radiation genes associated to E1 and I1p is observed in the Figure 4.

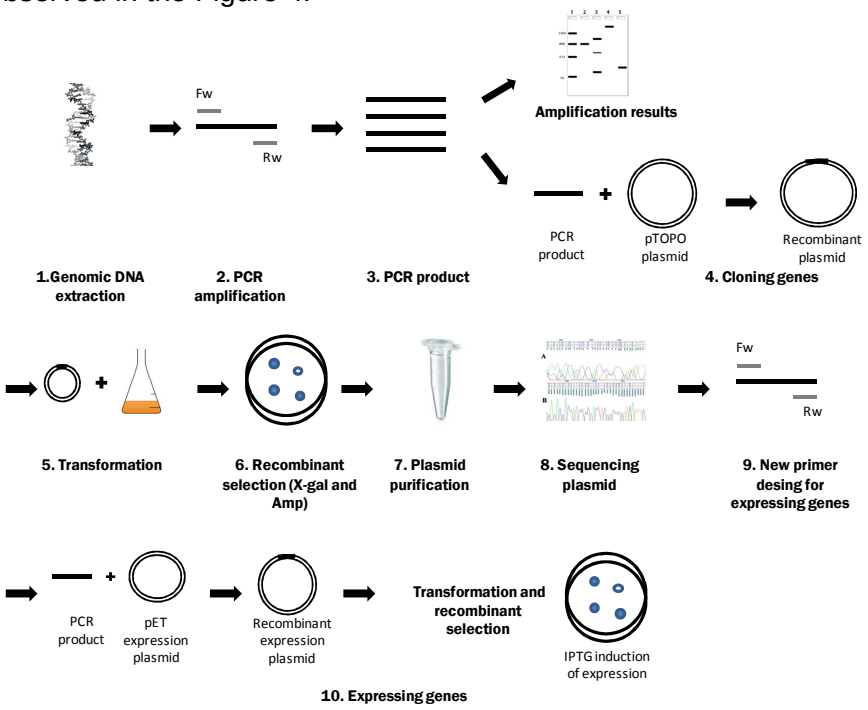


Figure 4. Description of the molecular strategy for cloning and expressing genes of *sod* and *cat* associated to E1.

1.3.1 Cloning *sod* gene

After PCR amplification of *sod* gene, we proceed to clone it with pTOPO cloning Kit. Once the recombinant plasmid was purified from *E. coli* DH5 alpha, samples were sequenced, in order to verify insertion, and sequence analysis (Figure 5).

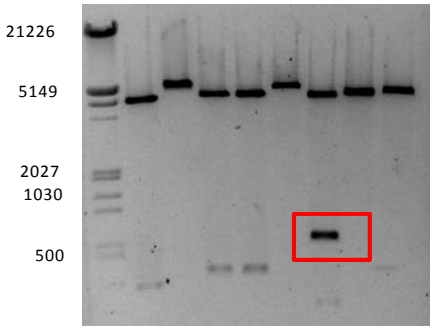


Figure 5. Agarose gel (1%) analysis of sod gene (630bp) in pTOPO after PCR amplification with specific primers for *sod* gene.

1.3.2 Expression of *sod* gene

The pET System has been developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in the pET-28a(+) vector (Figure 6) under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein, few hours after induction. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host (*E.coli* DH5-alpha), target protein expression may be initiated by transferring the plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lac*UV5 control (*E.coli* BL21). The expression is induced by the addition of IPTG to the bacterial culture.

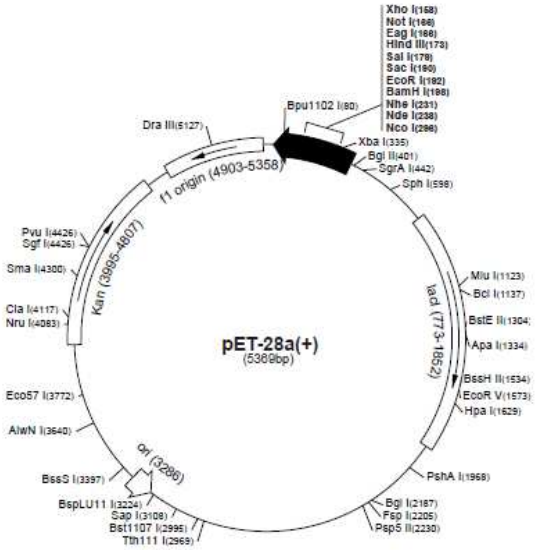


Figure 6. pET-28a(+) (5369 bp) vector structure. The most important elements are described; T7 promoter (370-386): Permits high levels of expression, N-terminal His tag that permits purification of recombinant fusion protein (270-287); Multiple cloning site (158-203); Kanamycin: resistance gene (3995-4807); pBR322: origin of replication (3286), that permits replication and maintenance in *E. coli.*

PCR amplification of *sod* gene with expression primers was performed and the amplified fragment was cloned into pET-28a(+). Construct was transformed into *E. coli* DH5 alpha in order to maintain its molecular stability. After insertion verification observed (Figure 5) and the obtained plasmid was purified.

Subsequently pET-28a(+) plasmid was transform into *E. coli* BL21, these cells are specially designed for expression of genes regulated by T7 promoter (Figure 6). The entire transformation reaction was cultivated in 10 mL of LB media containing kanamycin as selective marker. pET expression system is inducible by IPTG. Induction assay was performed during 16 hours of incubation with and without IPTG (1 mM) at 23ºC and 37ºC in LB at 140 rpm. SDS–PAGE (12%) gel was performed for recombinant *E. coli* BL21/IPTG induction (Figure 8), superoxide dismutase molecular weight is 22,8 kDa, and the molecular weight of the superoxide dismutase protein with the N terminal Histidine Tag is 26.6 kDa (Figure 7).

A) Protein sequence

```
MPFELPALPYPYDALEPHIDKETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSN
LEALPESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELAEAINKKFGSFAAFKDEFS
KAAAGRFGSGWAWLVVNNGELEITSTPNQDSPIMEGKTPILGLDVWEHAYYLKYQNRRPE
YIAAFWNIVNWDEVAKRYSEAKAK
```

B) Protein sequence with N terminal Tag

```
         10         20         30         40         50         60
MGSSHHHHHH SSGLVPRGSH MASMTGGQQM GRGSEFMPFE LPALPYPYDA LEPHIDKETM

         70         80         90        100        110        120
NIHHTKHHNT YVTNLNAALE GHPDLQNKSL EELLSNLEAL PESIRTAVRN NGGGHANHSL

        130        140        150        160        170        180
FWTILSPNGG GEPTGELAEA INKKFGSFAA FKDEFSKAAA GRFGSGWAWL VVNNGELEIT

        190        200        210        220        230        240
STPNQDSPIM EGKTPILGLD VWEHAYYLKY QNRRPEYIAA FWNIVNWDEV AKRYSEAKAK
```

Figure 7. Superoxide dismutase protein sequence. **A)** SOD protein sequence from E1 (22,8 kDa)**. B)** SOD protein sequence with the N terminal Histidine Tag (26.6 kDa).

In Table 1 there is the abbreviations used in figure 8, 10 and 11.

TC	Total cells extract
CFE	Total cells extract centrifugated
NI	Non induced cells
I	Induced cells

Table 1. Abbreviations used in figure from 8, 10 and 11.

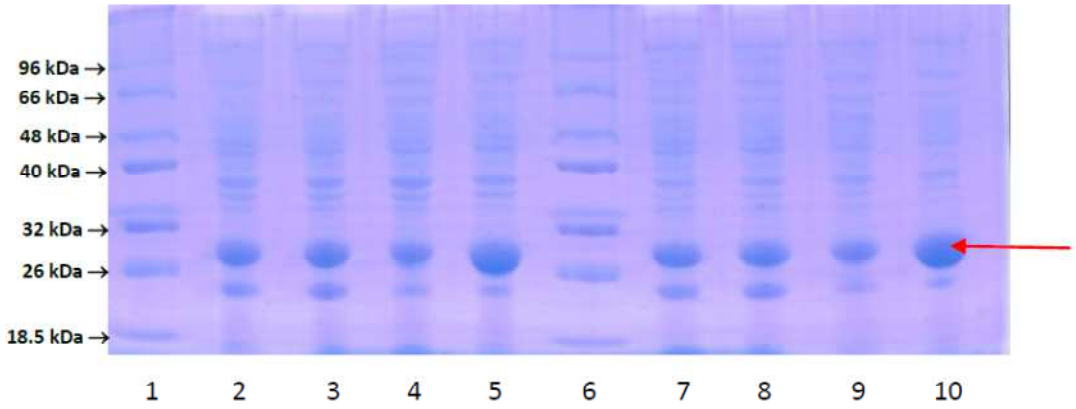


Figure 8. SDS–PAGE (12%) of Recombinant *E. coli* /IPTG induction. Lane 1, markers; Lane 2, 23°C NI TC; Lane 3, 23°C I TC; Lane 4, 37°C NI TC; Lane 5, 37°C I TC; Lane 6, markers ; Lane 7, 23°C NI CFE; Lane 8, 23°C I CFE; Lane 9, 37°C NI CFE; Lane 10, 37°C I CFE.

In addition, after *sod* expression was confirmed by SDS-PAGE, we proceed with the recombinant protein purification, by IMAC Purification of Polyhistidine-tagged Protein (Figure 9).

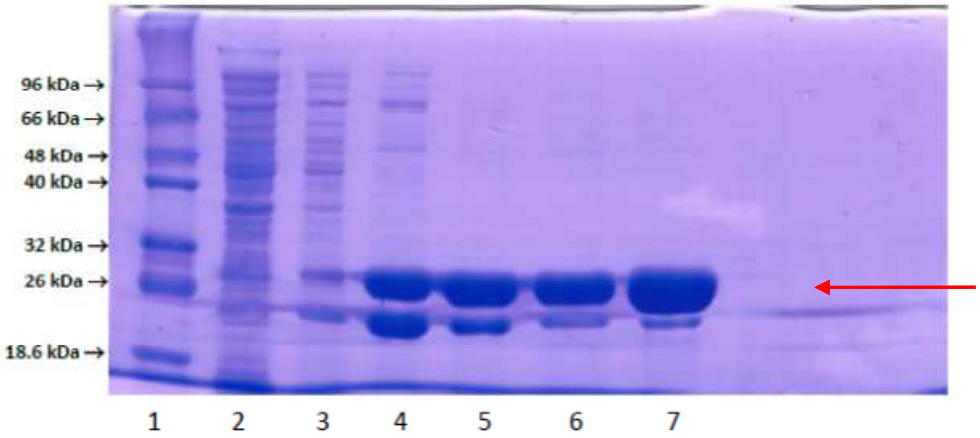


Figure 9. SDS–PAGE (12%) analysis of IMAC purification of recombinant SOD from E1 into *E. coli* BL21. Lane 1, markers; Lane 2, flow through; Lane 3, 10 mM imidazole buffer wash fraction; Lane 4, 50 mM imidazole buffer wash fraction; Lane 5, 100 mM imidazole buffer wash fraction; Lane 6, 250 mM imidazole buffer wash fraction; Lane 7, 500 mM imidazole buffer wash fraction.

The purified extract did not have SOD activity, probably the reason was that the His-tag interfered in the correct protein folding.

Re-cloning without N terminal His6-tag

New primers were designed (Table 2) to remove the Tag by removing *EcoR*I site and replacing with *Nde*I and then placing into pET22b (Figure 10) plasmid rather than pET28a

plasmid. In Figure 11 is observed that after the remotion of the His-Tag (pET22b) the expression is similar to the one observed in the His-tag recombinant protein (pET28a).

Primer	Sequence
SOD *NdeI*	TGCCATAGCATATGCCGTTTGAACTGC
SOD *XhoI*	TGCCATAGCTCGAGTTATTATTTAGC

Table 2. Primers designed for removing the His-tag.

The pET-22b(+) vector carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below (Figure 10).

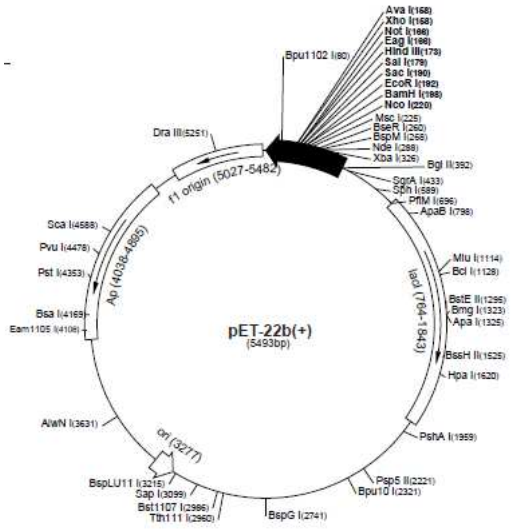
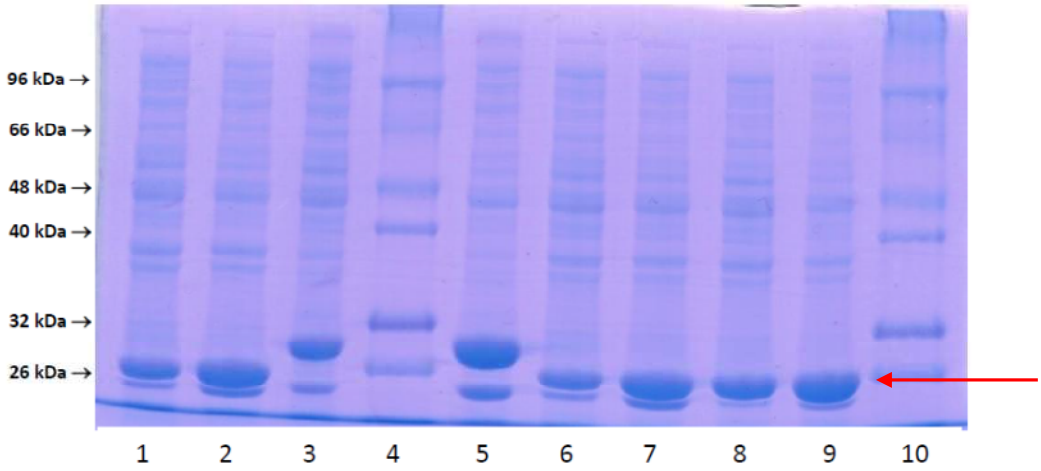


Figure 10. pET-22b(+) (5493 bp) vector structure. The most important elements are



described; T7 promoter (361-377): Permits high levels of expression, pelB coding sequence (224-289); Multiple cloning site (158-225); ampicillin: resistance gene (4038-4895); pBR322: origin of replication (3277), that permits replication and maintenance in *E. coli.*

Figure 11. SDS–PAGE (12%) of Recombinant *E. coli* /IPTG induction His-tag remotion. Lane 1, SOD/pET22b 20ºC NI TC; Lane 2, SOD/pET22b 20^{o}C I TC; Lane 3, SOD/pET28a 20^{o}C NI CFE; Lane 4, markers; Lane 5, SOD/pET28a 20^{o}C I CFE; Lane 6, SOD/pET22b 20ºC NI CFE; Lane 7, SOD/pET22b20^{o}C I CFE; Lane 8, SOD/pET22b 37^{o}C NI CFE; Lane 9, SOD/pET22b 37^{o}C I CFE; Lane 10, markers.

Heat treatment of CFE from recombinant SOD-E1 pET22b construct.

In order to test the thermal stability of the recombinant enzyme, 100 µL aliquots of CFE were incubated at 50℃, 60℃, and 70℃ for 10 min. They were then centrifuged. The supernatant was then analyzed on SDS-PAGE (Figure 12). From the gel a 65℃ treatment was chosen for the large scale purification SOD. The purified material was then stored in 3.2 M ammonium sulphate.

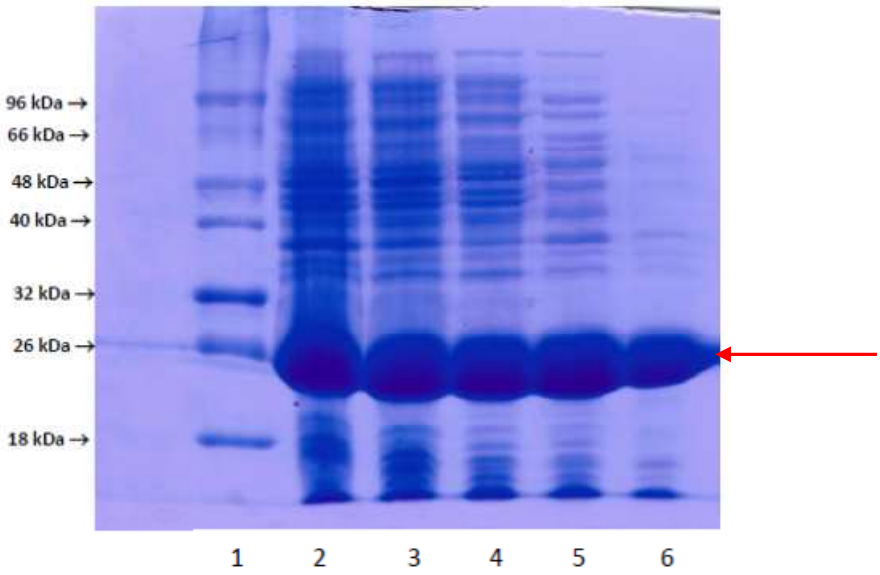


Figure 12. SDS–PAGE (12%) analysis of heat treatment of recombinant SOD. Lane 1, markers; Lane 2, TC; Lane 3, CFE; Lane 4, CFE heated 50ºC; Lane 5, CFE heated 60ºC; Lane 6, CFE heated 70ºC.

1.3.3 Molecular characterization of the Recombinant SOD from E1.

As part of the biochemical analysis of the SOD recombinant protein under study, we determined the optimal temperature and thermostability.

SOD activity was assayed by the method of Winterbourn *et al.* based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide. One unit was defined as the amount of enzyme causing one half of the maximum inhibition of NBT reduction. The graph shows that the enzyme is active at a broad temperature range (Figure 13), from 30 to 80℃, a non common property. The enzymatic activity was affected only when the enzyme was exposed to temperatures over 70℃ and less than 30ºC. This result opens a wide perspective of biotechnological applications, because this enzyme could be used under diverse industrial conditions and in a broad range of temperatures.

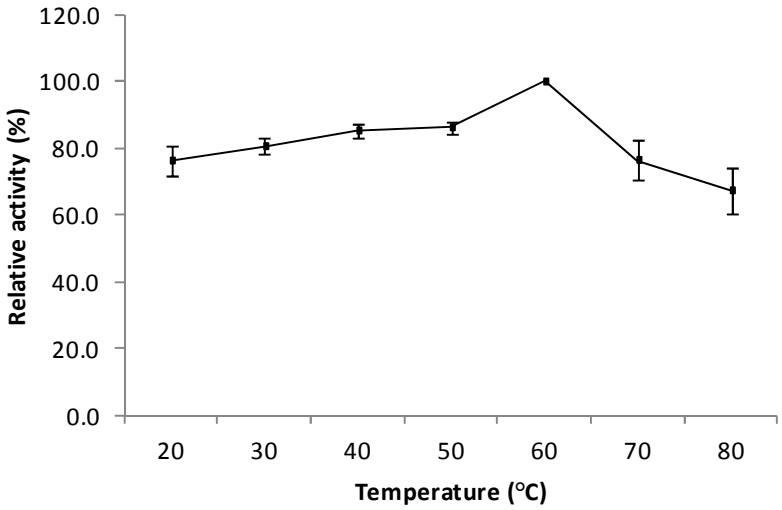


Figure 13. Superoxide dismutase optimal temperature of recombinant protein.

We also analyzed the thermostability of the recombinant SOD from E1 at 60℃. The enzymatic activity observed was maintained nearly unchanged (Figure 14) for 10 h, even though after 16 h of incubation the specific activity decreased 50%, and it remains constant for 43 h. This results confirm the high biotechnological potential of this enzyme because its thermostability.

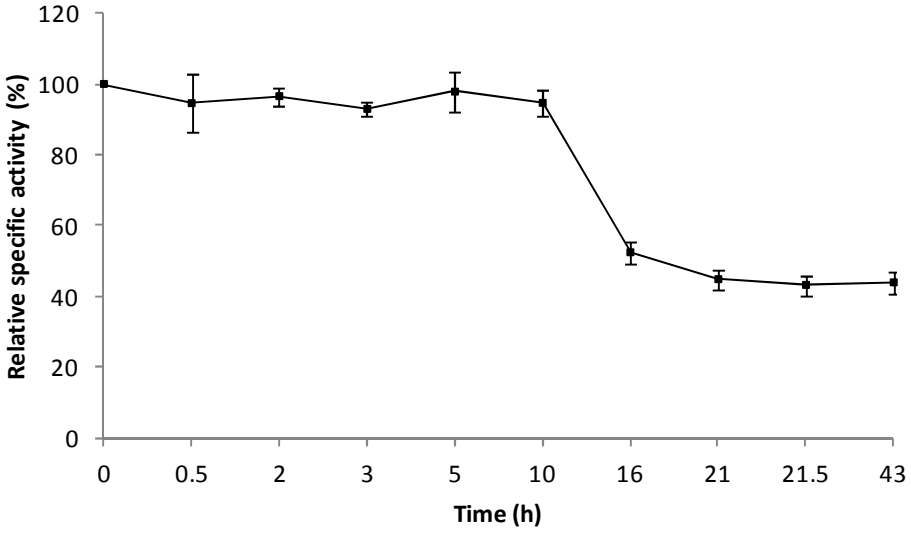


Figure 14. Thermostability of recombinant SOD from E1 expressed in *E. coli* BL21 at 60℃.

UV resistance

E.coli cells were routinely grown in LB medium at 37ºC with shaking. The cells were grown to OD_{600} = 0.4 and immediately were exposed to UV radiation.

The microorganism containing the recombinant SOD and the *E.coli* BL21 were exposed for 0-35 min to UV-Radiation in a UV chamber with a UVC lamp of 2.8 W/m^2. UV dosage

was measured using radiometer in J/cm^2. The doses of UV radiation applied to the cultures and its intensity are shown on Table 3. The type of UV radiation used along the experiments was UVC which is considered the germicidal type of UV radiation.

Time of exposure (min)	UV dosage (J/cm^2)
0	0
5	0.168
10	0.324
15	0.533
20	0.738
25	0.938
30	1.136
35	1.331

Table 3. Exposure times and total energy.

After the treatment with UVC the cells were transferred and incubated in solid media at the optimal temperature until they generated colonies. Then, the colonies were counted and viability rate was calculated and expressed as percent of viable cells with respect to the non-irradiated cultures (Figure 15).

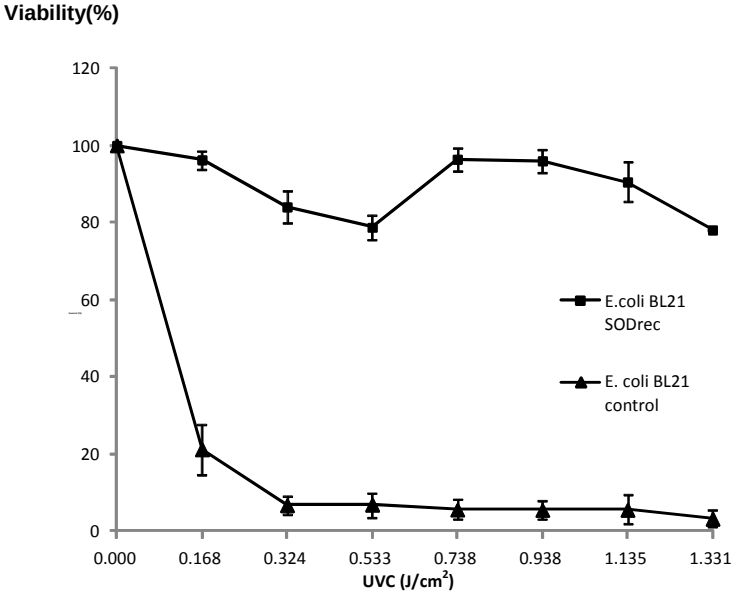


Figure 15. Viability of the cells exposed to UV radiation. *E.coli* BL21 control without the plasmid that contains E1*sod* gene (square), *E.coli* BL21 recombinant with the plasmid that contains E1*sod* gene (triangle). Viability was expressed in percent.

The results obtained from the previous experiment show that superoxide dismutase from E1 is a very efficient enzyme capturing reactive oxygen species since the recombinant *E. coli* BL21 cells were able to survive the exposure to UVC in comparison to the *E.coli* BL21 which do not contain the plasmid with the E1 *sod* gene.

A) *E. coli* BL21 SOD rec B) *E. coli* BL21 control

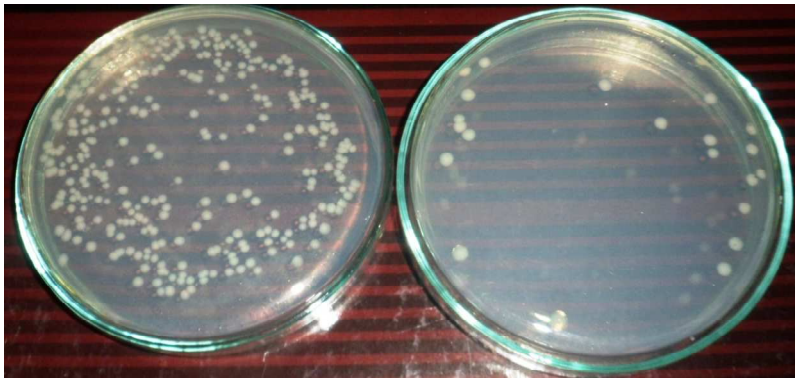


Figure 16. *E. coli* BL21 cells exposed to UVC during 35 min. A) *E. coli* BL21 cell containing the recombinant SOD. B) *E. coli* BL21 without the insertion of the recombinant plasmid. This picture shows the effect of UVC on the culture of *E. coli* BL21 without and with the plasmid containing E1 *sod* gene. Observe that the number of colonies present on the recombinant *E.coli* culture is much higher than the one present in the control plate indicating the strong protecting effect on UVC conferred by the incorporation of *sod* gene into the *E. coli* BL21 culture.

2. Cloning and expression of the gene that codify for catalase from I1P.

The experimental strategy designed for achieving this objective is explained in the Figure 17. The aim was to clone and express the gene that codify for catalase in the microorganism I1P.

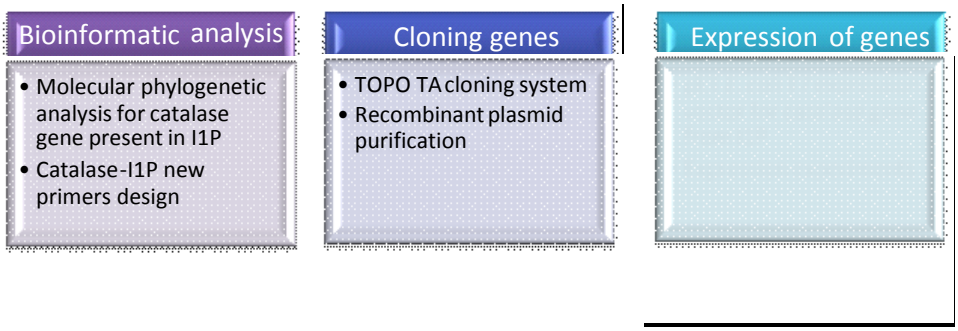


Figure 17. Experimental strategy for cloning and expression.

2.1 Bioinformatics analysis of catalase sequence from I1P.

In the case of I1P to obtain the catalase sequence, we performed bioinformatic analysis and designed new primers from the sequences available in online database of microorganism who were close related to I1P (Table 4).

Primer	Sequence	Type
CAT FW1	ATCAGGACTGGTGGCCCAAC	F
CAT IW1	AGGCCATACGAATGAACAGG	R
CAT RIW1	GTGTTCGAAGGCCAGGATAG	F
CAT RW1	TTAGGCCAGATCAAAACGATC	R
CAT FW2	ATGAATCAGCGGGTATTAGTAAG	F
CAT IW2	CTGAATCAAATGCCCATCCT	R
CAT RIW2	AAACCGCAGCAGTCTGATCT	F
CAT RW2	TTAATCTTTCAGCTTGTCGGCC	R
CAT FW3	ATGAGCAAGAAAGGACTGACCAC	F
CAT IW3	GTGGGTGACGGTGAAAGTG	R
CAT RIW3	GGAATCACCGTGAAGACGAT	F
CAT RW3	TTATTTCAGACCTAACGCCTTGG	R

Table 4. Primers designed for catalase PCR amplification from I1P.

2.2 Cloning gene

After PCR amplification of *catalase* gene, we proceeded to clone it with pTOPO cloning Kit (Figure 18). Once the recombinant plasmid was purified from *E. coli* DH5 alpha, samples were sequenced, in order to verify insertion, and sequence analysis.

After several trials we could obtain the catalase sequence. The catalase gene from I1P has 1437 nucleotides. The protein contains 478 amino acid residues and the protein molecular weight predicted was 54.93 kDa. This information is in agreement with results obtained for the native catalase purified from I1P (Figure 19), these results were included in the previous technical report.

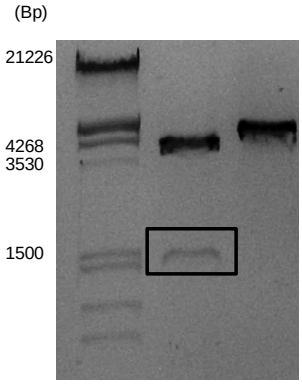


Figure 18. Agarose gel (1 %) analysis of catalase gene (1449 bp) in pTOPO after a PCR amplification with specific primers for catalase gene.

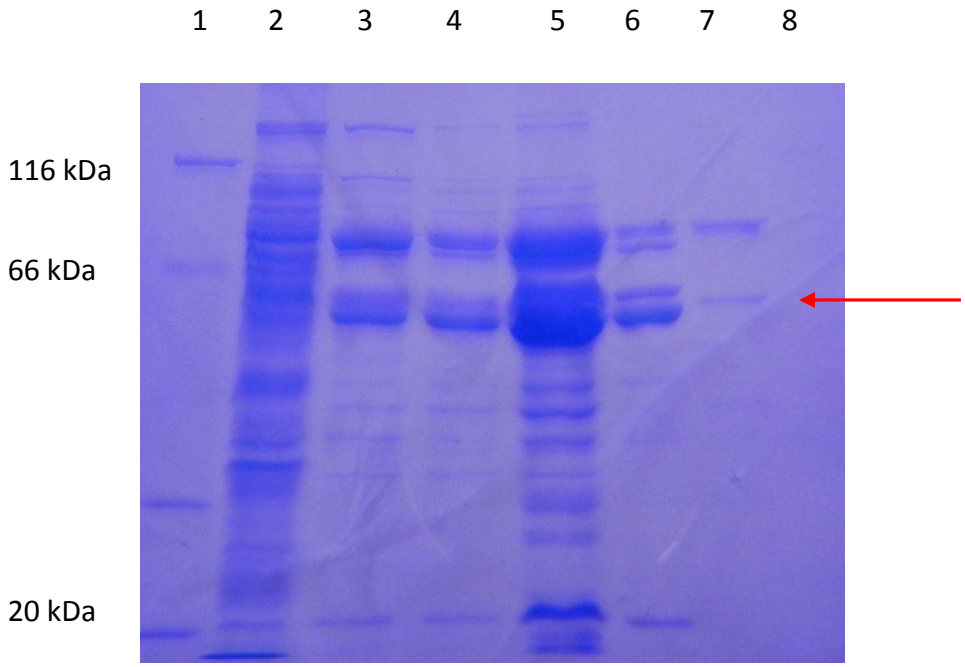


Figure 19. SDS-PAGE gel (12 %) analysis of catalase protein purification (55 kDa). Lane 1, markers; Lane 2, crude extract; Lane 3, DEAE I-DEAE 12; Lane 4, DEAEII-DEAE-3; Lane 5, QHT1 (41-43%); Lane 6, Superdex Cat peak; Lane 7, Superdex peak 4.

NATIVE Protein sequence

```
        10         20         30         40         50         60
MSKKGLTTAA GAPVVDNNNV ITAGKRGPML LQDVWFLEKL AHFDREVIPE RRMHAKGSGA

        70         80         90        100        110        120
YGTFTVTHDI SQYTRAKIFS EIGKQTDMFI RFSTVAGERG AADAERDIRG FAMKFYTEEG

       130        140        150        160        170        180
NWDLVGNDTP VFYLRDPLKF PDLNHVVKRD PRTNLRNPTY KWDFFSLSPE SLHQLTIDFS

       190        200        210        220        230        240
DRGIPKSYRH MHGFGSHTFS FINANNERFW VKFHFRCEQG IENLMDDEAE AIIAKDRESS

       250        260        270        280        290        300
QRDLYEAIER GDFPRWKLQI QIMPEHEASQ TPYNPFDLTK VWPHGDYPLI DVGFFELHRN

       310        320        330        340        350        360
PENYFSEVEQ MAVNPANVVP GVSFSPDKML QGRLFSYGDA QRYRLGVNHH QIPVNSPKCP

       370        380        390        400        410        420
FHNYHRDGAM RVDGNSGNGA TYEPNSFGLF QEQPDFSEPP LSIEGAADHW NHREDDDYYS

       430        440        450        460        470
QPRALFNLLS AEEHQRMFTR IAGELSQVPE QIQRRQVDLF SKVHPNYGAG VAKALGLK
```

Figure 20. Catalase native protein sequence. Catalase aminoacid sequence (55 kDa).

2.3 Expression analysis

PCR amplification of *catalase* gene with expression primers was performed and the amplified fragment was cloned into pET-28a(+) (Figure 6). Construct was transformed into *E. coli* DH5 alpha in order to maintain its molecular stability. Subsequently pET-28a(+) plasmid was transform into *E. coli* BL21. The entire transformation reaction was inoculated in 10 mL of LB media containing kanamycin as selective marker. pET expression system is inducible by IPTG. Induction assay was performed during 16 hours of incubation with and without IPTG (1 mM) at 16ºC and 28ºC in LB at 140 rpm. SDS–PAGE (12%) gel was performed for recombinant *E. coli* BL21/IPTG induction (Figure 21), catalase molecular weight is 55 kDa, and the molecular weight of the catalase protein with the N terminal Histidine Tag is 57 kDa.

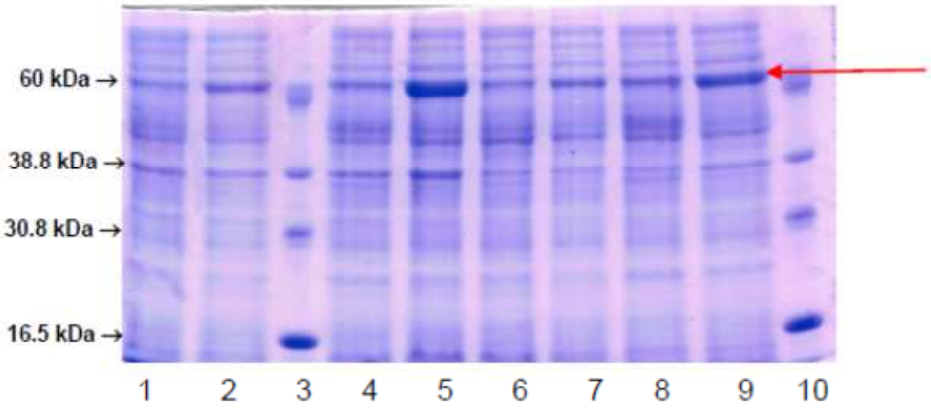


Figure 21. SDS-PAGE (12 %) analysis of soluble expression levels of catalase/28a from I1P; 57 kDa). Lane 1, 16ºC UI TC; Lane2, 16ºC I TC; Lane 3, markers; Lane 4, 28ºC UI TC; Lane 5, 28ºC I TC; Lane 6, 16ºC UI CFE; Lane 7, 16ºC I CFE; Lane 8, 28ºC UI CFE; Lane 9, 28ºC I CFE; Lane 10, markers (see table 1 for abbreviations used in figure 21 and 22).

In addition, after *catalase* expression was confirmed by SDS-PAGE, we proceeded with the purification of the recombinant protein using a IMAC Purification of Polyhistidine-tagged Protein (Figure 22). The CFE was loaded onto an equilibrated 10 mL gravity fed column. The purification was conducted on a step gradient basis where the concentration of imidazole was increased from 10mM to 500mM along the purification. Activity assays were conducted to ensure protein preservation. The protein was maintained active in 3.2 ammonium sulphate.

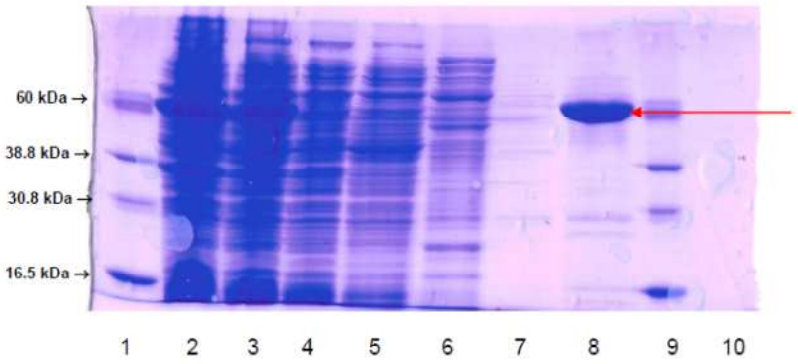


Figure 22. SDS-PAGE (12 %) analysis of IMAC purification fractions catalase/28a from I1P; 57 kDa. Lane 1, markers; Lane2, catalase/28a TC; Lane 3, catalase/28a CFE; Lane 4, , catalase/28a Flow-through; Lane 5, 10 mM imidazole elution; Lane 6, 50 mM imidazole elution; Lane 7, 500 mM imidazole initial elution; Lane 8, catalase/28a 500 mM imidazole elution; Lane 9, markers.

2.4 Molecular characterization of the Recombinant CAT from I_1P.

For the biochemical characterization of the recombinant protein under study, we determined the optimum pH, optimum temperature and thermostability.

Catalase activity was assayed by the method of Beers *et al*. The test for activity consisted in measuring the decomposition of H_2O_2 by the action of catalase enzyme. One unit of activity is defined as the amount of enzyme that catalyzes the decomposition of 1 µmol of H_2O_2 per minute.

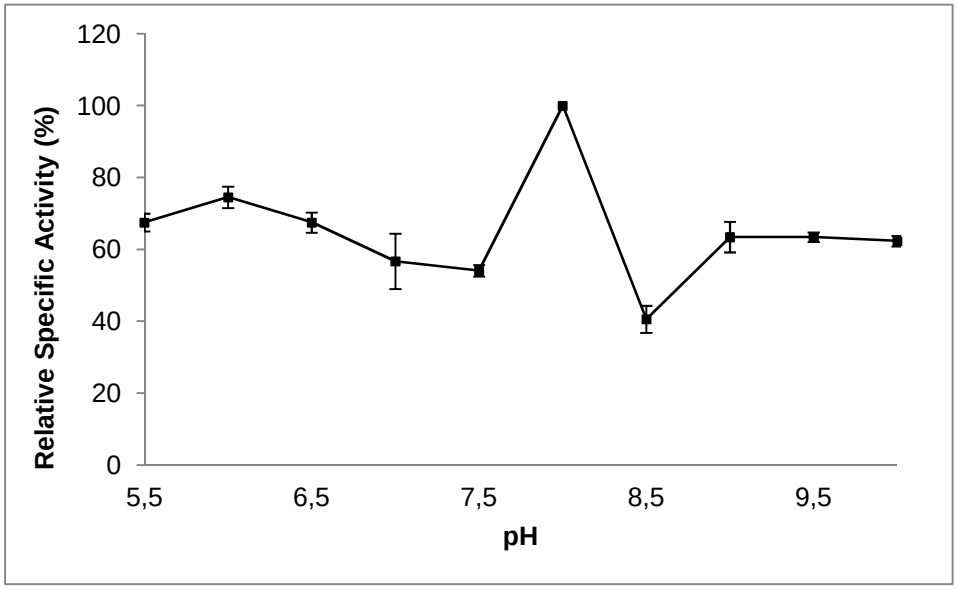


Figure 23. Optimum pH of recombinant catalase from I_1P.

Figure 23 shows that the optimum pH of the recombinant catalase is 8. Nevertheless, it also shows two additional minor peaks at pH 6 and 9.

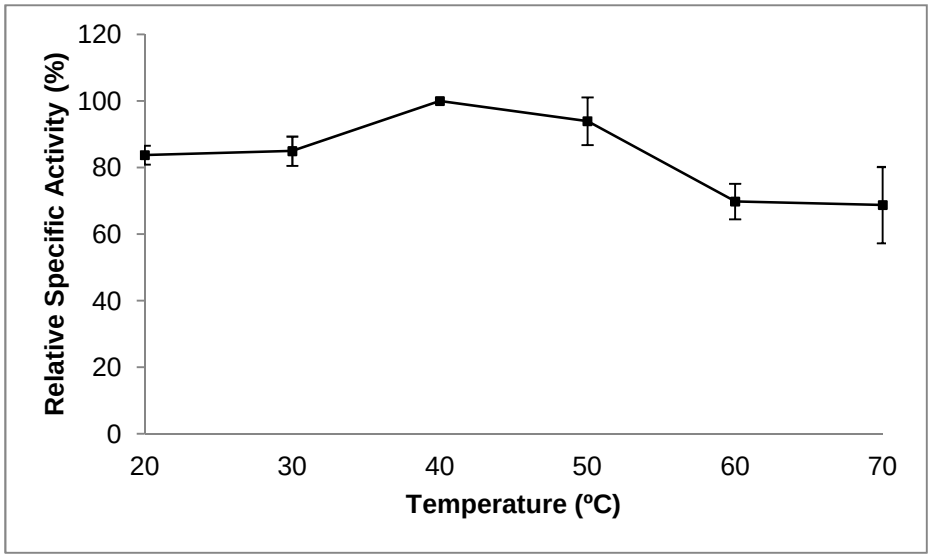


Figure 24. Optimum temperature of recombinant catalase from I_1P.

The recombinant catalase showed an optimum temperature of 40ºC (in Figure 24). Furthermore, this figure also shows that the enzyme remains active in a range between

20ºC and 70ºC, indicating that the enzyme can catalyze the reaction along a wide range of temperatures.

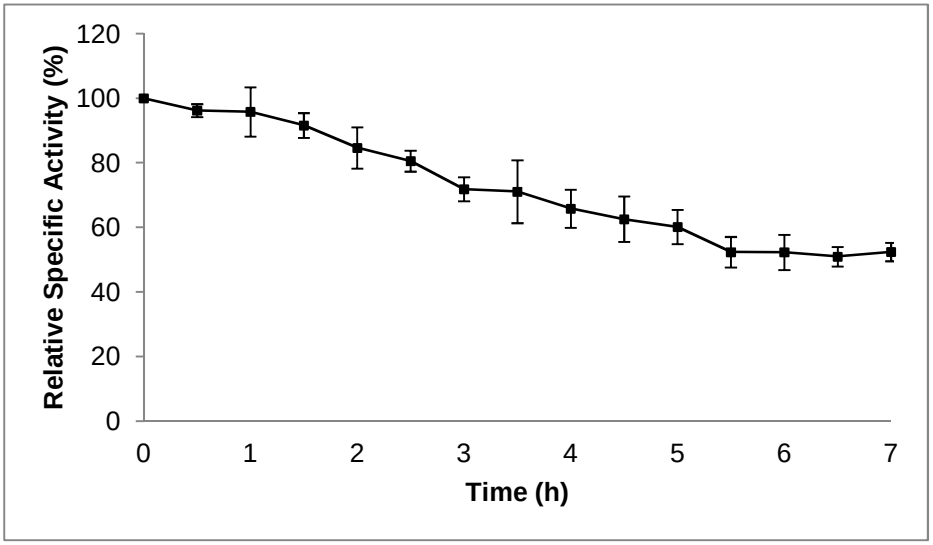


Figure 25. Thermostability of recombinant CAT from I_1P expressed in *E. coli* BL21(DE3) at 50℃.

Once, optimum pH and temperature were performed, Thermostability of the recombinant protein was analyzed. The graph shows that the enzyme activity decreases approximately to 50% of its initial activity in 6 hours.

2.5 Catalase UV resistance

E.coli cells were routinely grown in LB medium at 23ºC with shaking at 140 rpm. The cells were grown to OD_{600} = 0.4 and immediately were exposed to UV radiation.

The microorganisms were exposed for 0-35 min to UV-Radiation in a UV Chamber with a UVC lamp of 2.8 W/m^2. UV dosage was measured using radiometer in J/cm^2. The doses of UV radiation applied to the cultures and its intensity are shown on Table 3. The type of UV radiation used along the experiments was UVC which is considered a germicidal type of UV radiation.

After the treatment with UVC the cells were transferred to solid media and incubated at the optimal temperature until they generated colonies. Then, the colonies were counted and viability rate was calculated and expressed as percent of viable cells.

The viability rate was determined and expressed as percent of counted viable cells with respect to the non-irradiated cultures (Figure 26).

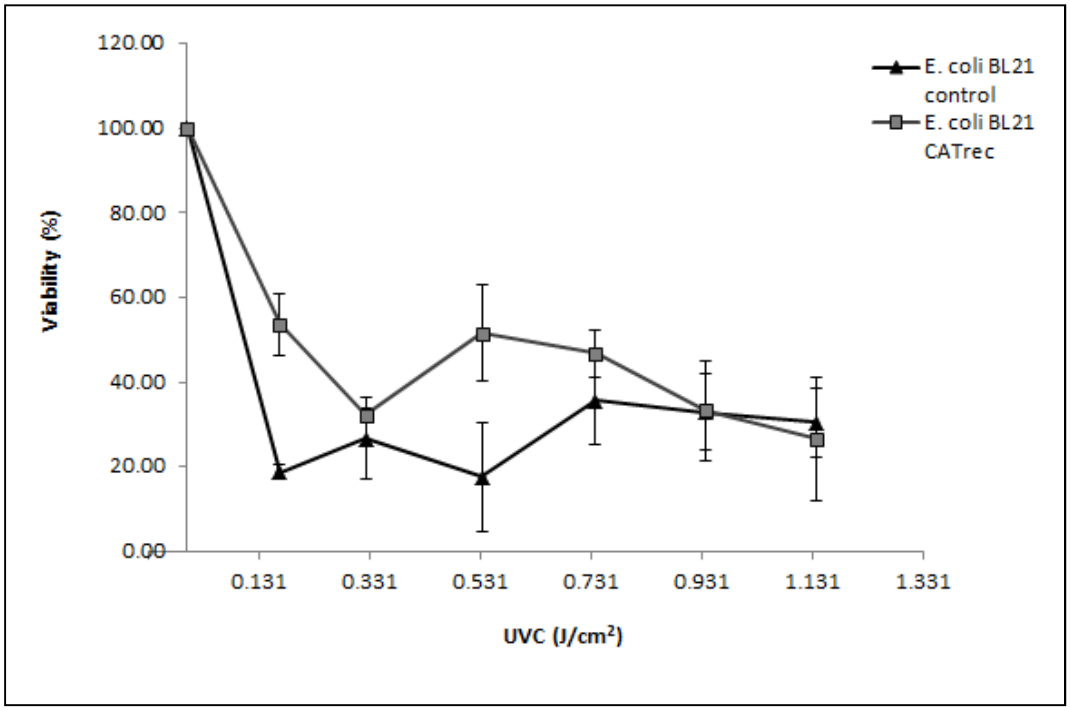


Figure 26. Viability of the cells exposed to UV radiation. *E.coli* BL21 control (triangle) and *E.coli* BL21 CAT recombinant (square).

The recombinant E.coli cells containing the CAT gene from I1P were able to protect the cells against UVC radiation but not as effectively as they were protected when the sod gene was incorporated.

A) *E.coli* BL21 control B) *E. coli* BL21 CAT rec

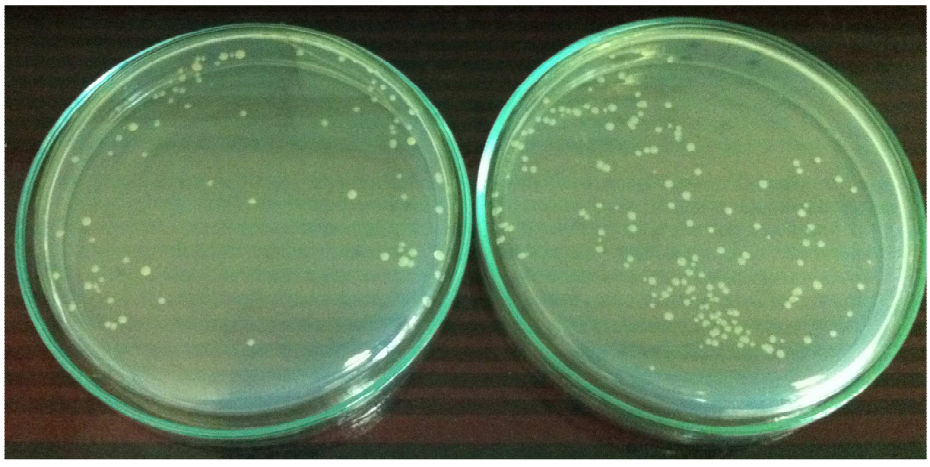


Figure 27. *E. coli* BL21 cells exposed to UVC during 35 min. A) *E. coli* BL21 without the recombinant plasmid (negative control). B) *E. coli* BL21 CAT recombinant.

The protection effect conferred by the incorporation of I_1P *cat* gene to *E.coli* exposed to UVC for 35 minutes was not very significant compared to the *E.coli* control without the recombinant plasmid. This means that the transformation of *E. coli* with the gene of I_1P catalase does not improve significantly the resistance of *E. coli* to UVC radiation.

Conclusion

We have been able to successfully clone and express into *Escherichia coli* the genes that codify for the enzymes superoxide dismutase and catalase from the selected microorganisms, E1 and I1P, to study protection effects of these genes when the *E. coli* strain is exposed to UV radiation.

From the experimental results obtained we have been able to conclude that the enzyme superoxide dismutase from the thermophile E1 is a very active enzyme and extremely efficient in its function as antioxidant by capturing superoxide radicals once the cells were exposed to inductors of free radicals such as UV radiation, specifically UVC.
The cloning and the expression of this gene into *E. coli* demonstrated the importance of this enzyme acting as a free radical scavenger. The incorporation of the *sod* gene from E1 into *E. coli* resulted in the protection of *E.coli* against UVC radiation exposure.
The recombinant protein was purified and several properties were studied. The recombinant SOD from E1 is a very thermostable enzyme maintaining 100% of its activity for nearly 10 hours a remarkable property for an enzyme when compared with its mesophilic counterparts. Additionally the recombinant SOD has an optimal temperature of 60ºC and an optimal pH of 7.5.

In the case of the I1P catalase we were also able to clone and express it into *E.coli* . But in this case even when the enzyme was successfully cloned and functionally expressed, it was able to protect in a less efficient manner *E.coli* cells against the effects of UVC radiation.

Several properties of the recombinant I1P protein were determined. The optimum pH was determined to be pH 8.0 meanwhile its optimal temperature for activity was 40ºC. The thermostability of the recombinant protein showed that the recombinant protein was able to maintain nearly 50% of its activity for more than five hours.

The successful functional incorporation of antioxidant genes *sod* and *cat* into a microorganism which did not have the resistance to UV radiation and the capacity of conferring protection against it, opens new avenues to understand and to look in more depth what makes one enzyme more effective than other in the protection against radiation.

Summary of completed work during the project

From the two microorganisms:

a) Microorganism 1 (strain E1)

- Biochemical characterization: Tº, pH, [NaCl], carbon sources
- Optimum growth conditions
- UV radiation studies

b) Microorganism 2 (strain I1p)

- Biochemical characterization: Tº, pH, [NaCl], carbon sources
- Optimum growth conditions
- UV radiation studies

c) Enzymatic work

- Purification of the enzyme SOD from E1
- Purification of the enzyme SOD from I1P
- Purification of the enzyme catalase from E1
- Purification of the enzyme catalase I1P

d) Molecular work

- Cloning of *sod* gene from E1 into *E. coli*
- Cloning of the *cat* gene from I1P into *E. coli*
- Expression of Mn superoxide dismutase gene from E1 into *E. coli*
- Expression of catalase gene from I1P into *E. coli*
- Molecular characterization of the superoxide dismutase recombinant enzyme.
- Molecular characterization of the catalase recombinant enzyme.

e) UV resistance studies

- UV resistance conferred to *E. coli* by the enzyme SOD from E1
- UV resistance conferred to *E. coli* by the enzyme SOD from I1P
- UV resistance conferred to *E. coli* by the enzyme catalase from E1
- UV resistance conferred to *E. coli* by the enzyme catalase from I1P

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Presentations in International Conferences

1. **Blamey, J.M.** "Extremophile Response for Protection against UV-Radiation Damage", Extremophiles, Key to Bioenergy The University of Georgia, Athens, GA, USA, September 2011.

2. Monsalves, M.T., Ollivet-Besson, G., **Blamey J.M.,** "Effect of ultraviolet radiation on the catalase and superoxide dismutase enzymatic system in a thermophile from the *Geobacillus* genus." Thermophiles 2011, Big Sky, Montana, USA, September 2011.

3. Chirino, B., Boehmwald, F, **Blamey J.M.**, "Molecular cloning and enzymatic characterization of thermophilic superoxide dismutase from *Geobacillus sp*." Thermophiles 2011, Big Sky, Montana, USA, September 2011.

4. Monsalves, M.T., Ollivet-Besson, Papić, L., **Blamey J.M.** "Optimization of the antioxidant activity of the enzyme superoxide dismutase from the thermophile E1 induced by ultraviolet radiation" Latin American Congress of Biotechnology. Santiago Chile, August 2011.

5. **Blamey, J.M.** "Incineration-proof hyperthermophiles from laboratory drying ovens" Forum of the Latin American Office for Scientific Research of AFOSR from USA. University of Santiago, Santiago Chile. October 2009.

6. **Blamey, J.M.** "Extreme Microorganisms and Enzymes from a Sterilization Oven" Forum Science & Industry on Extremophiles Valais, Sion, Switzerland March 2009.

Papers

1. Monsalves S. M.T., Amenábar, M.J., Ollivet-Besson R.G.P., **Blamey, J. M** (2012) Effect of UV radiation on a thermostable superoxide dismutase purified from a thermophilic microorganism isolated from a sterilization drying oven. Protein and Peptide Letters. (in press)

2. Chirino, B., Boehmwald, F, **Blamey J.M.** (2012) Molecular cloning, expression and characterization of a thermophilic manganese superoxide dismutase from a thermophile isolated from a sterilization drying oven. PLOS one (to be submitted)